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Veterinary Parasitology 110 (2002) 11–15

veterinary parasitology

www.elsevier.com/locate/vetpar

First Portuguese isolate of *Neospora caninum* from an aborted fetus from a dairy herd with endemic neosporosis

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Received 6 December 2001; received in revised form 25 September 2002; accepted 6 October 2002

Abstract

Neospora caninum was isolated from the brain of an aborted 4-month-old fetus from a dairy cow herd with endemic neosporosis in Porto, Portugal. The fetal brain homogenate was inoculated interperitoneally first into outbred Swiss Webster mice given dexamethasone and then the peritoneal exudates from these mice was co-inoculated with mouse sarcoma cells in the peritoneal cavity of mice given dexamethasone. *N. caninum* tachyzoites were seen in peritoneal exudate of the second passage. Tachyzoites from the peritoneal exudate reacted positively with anti-*N. caninum* antibodies and not with anti-*Toxoplasma gondii* antibodies and contained *N. caninum* specific DNA. This Portuguese isolate of *N. caninum* has been successfully maintained in cell culture. The dam of the aborted fetus had an antibody titer of 1:10240 in the *Neospora* agglutination test (NAT). Antibodies to *N. caninum* were found in 76 of 106 cows from this herd in titers of 1:40 in 31, 1:80 in 22, ≥1:160 or more in 23 in the *Neospora* agglutination test. This is the first isolation of a viable *N. caninum*-like parasite from any host in Portugal.

Keywords: *Neospora caninum*; Abortion; Isolation in mice; Antibodies; *Neospora* agglutination test; Cattle; Portugal

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PII: S0304-4017(02)00333-3

1. Introduction

Neospora caninum is a major cause of abortion in cattle in many countries. The diagnosis of *N. caninum*-induced abortion is difficult. For a definitive diagnosis of bovine neosporosis, fetal examination is necessary because up to 90% of cattle may have *N. caninum* antibodies, and many calves are born congenitally-infected but clinically normal (Dubey and Lindsay, 1996; Dubey, 1999). Although demonstration of *N. caninum*-specific antibodies in the fetus is indicative of transplacental infection, a negative serologic examination does not rule out infection. Therefore, demonstration of *N. caninum* infection in the fetus is the best available evidence for *N. caninum*-induced abortion. For demonstration of *N. caninum*, immunohistochemical staining of fetal tissues with *N. caninum*-specific antibodies is the most commonly used method (Dubey and Lindsay, 1996). The detection of *N. caninum* DNA in fetal tissues has not yet become routine (Dubey, 1999). Attempts to isolate viable *N. caninum* from bovine fetuses has been difficult, insensitive, and expensive. For example, in one study, *N. caninum* was isolated from only two of more than 100 fetuses suspected to have neosporosis (Conrad et al., 1993). One reason for this difficulty in recovering viable *N. caninum* is because most *N. caninum* in aborted bovine fetuses are dead, and the sensitivity of the cell culture to isolate *N. caninum* from autolysed bovine fetuses is probably low (Conrad et al., 1993; Dubey and Lindsay, 1996). Therefore, there are only a few isolates of *N. caninum* from bovine fetuses available worldwide, and little is known of their antigenic variability (Conrad et al., 1993; Yamane et al., 1997; Stenlund et al., 1997; Davison et al., 1999; Kim et al., 2000; McAllister et al., 2000). In the present paper, we describe isolation of the first strain of *N. caninum* from a dairy cattle fetus in Portugal by bioassays in immunosuppressed mice co-infected with mouse sarcoma cells.

2. Materials and methods

2.1. Dairy herd with endemic neosporosis

The herd consisted of 106 adult Holstein cows located in Porto district, northern Portugal. The cows were barn-housed year-round, milked two times per day, and were fed total mixed rations, corn silage and straw. Average milk production was 6941 kg in 305 days. Replacement heifers were raised on the farm. Breeding was exclusively by artificial insemination with frozen semen, and all cows were vaccinated against bovine herpesvirus type-I (IBR), bovine diarrhea virus (BVD), and leptospirosis. Blood samples were collected from all adult cows by tail venipunture. Blood samples were left to clot, centrifuged and the serum separated and frozen at 20 °C until used for antibody determination.

2.2. Serologic examinations

The *Neospora* agglutination test (NAT) as described by Romand et al. (1998) was used to detect *N. caninum* antibodies. Sera were analyzed for *T. gondii* antibodies using the *Toxoplasma* direct agglutination test of Desmonts and Remington (1980).

2.3. Isolation of Neospora caninum

A portion of brain of a 4-month-old fetus from a dairy cow from this herd was homogenized in 2% (v/w) phosphate buffered saline (PBS)-trypsin (Difco, Detroit, USA) and incubated in a shaker bath at 37 °C for 1 h. This brain suspension was washed with 50 ml of PBS three times by centrifugation at 1062 g for 10 min. The pellet was resuspended in 4 ml of PBS, and approximately 1 ml of the suspension was inoculated intraperitoneally (i.p.) into four Swiss Webster mice previously given10 μg/ml dexamethasone (Keia Laboratória, Hoogstrated, Belgium) in drinking water ad libitum for 10 days, as described by Romand et al. (1998). Four days after the i.p. inoculation, mice were sacrificed and their peritoneal cavity was washed with 5 ml of PBS. The peritoneal wash was mixed with 2 ml mouse sarcoma cells Tg 180 (2×10^5 cells/μl) (Desmonts and Remington, 1980). The mixture was then centrifuged for 10 min at 1062 g. The pellet was resuspended in 4 ml of PBS, and approximately 1 ml of this suspension was inoculated i.p. into four mice given dexamethasone. Four days later, these mice were sacrificed and their peritoneum was washed with 5 ml of PBS. The washing fluids were incubated with 1% PBS-trypsin at 37 °C on a shaker. The mixture was centrifuged three times with PBS at 1062 g for 10 min. The pellet was immediately used for immunofluorescence assay.

2.4. Detection of N. caninum tachyzoites by indirect fluorescent antibody test (IFAT)

The IFAT was performed in 10 circles of immunofluorescence slides prepared at the University of Porto laboratory. For this, 50 μl of the pellet obtained as previously described were placed in each circle and dried in an incubator at 37 °C. After drying, the slides were fixed with 50% methanol for 30 min at 22 °C. After fixation, 50 μl of anti-*N. caninum* rabbit antiserum (Lindsay and Dubey, 1989a) diluted 1:5000 in PBS, was placed in each circle. The slides were then incubated at 37 °C for 1 h in a humidified atmosphere. After incubation, the slides were washed three times in PBS for 30 min. Finally, 50 μl of a goat anti-rabbit fluorescein isothiocyanate (FITC) conjugated IgG antibody (Sigma, St. Louis, USA) diluted at 1:160 in PBS with 0.05% of Evans Blue (George T. Gurr, London, UK) was added to each circle on the slides. Slides were then incubated at 37 °C for 30 min in a humidified atmosphere, and washed 3 times in PBS for 10 min. Immediately after washing, slides were observed in an epifluorescence microscope (Nikon Optiphot, Japan) at ×200 and ×400 magnifications, with a B-2 A (450–520 nm) filter. Tachyzoites of the NC 1 strain of *N. caninum* were used as positive control. For the negative control, the same parasites were used but incubated with anti-*Toxoplasma gondii* rabbit antiserum (Lindsay and Dubey, 1989a).

2.5. Genetic characterization of N. caninum isolate

Genomic confirmation of *N. caninum* isolate was made on mouse ascitic fluids 4 days p.i. after DNA extraction and amplification by polymerase chain reaction (PCR). The PCR for detection of *N. caninum* was performed on a LightCycler Instrument (Roche Diagnostics, Meylan, France), according to the method described by Müller et al. (2002), using primers targeted to the repetitive genomic Nc5 region. For this, whole DNA from 100 μl of mouse

peritoneal fluid containing tachyzoites was extracted and purified using the High Pure PCR Template Preparation kit (Roche Molecular Biochemicals, Meylan, France) according to the manufacturer's recommendations. This method allows a real time amplification and detection of specific amplification products by fluorescent hybridization probes. The MRC5 cell culture of *N. caninum* strain (NC-1) was used as positive controls for the PCR assay.

3. Results

Neospora caninum-like tachyzoites were first seen microscopically in peritoneal exudate from mice inoculated with brain homogenate from the bovine fetus and subsequently in peritoneal exudate of mice co-infected with mouse sarcoma cells. These tachyzoites reacted with anti-*N. caninum* antibodies but not with anti-*T. gondii* antibodies in the IFAT. The isolate of *N. caninum*-like parasite in the present study (designated NC-Porto1) has been successfully maintained in cell culture for seven months, further studies are planned to characterize the NC-Porto 1 strain. The sample of mouse ascitic fluid containing tachyzoites showed amplification products by PCR, specific of *N. caninum*, thus providing a definitive genomic identification of the isolate. Additionally, the same sample was negative by PCR targeted to the respective B 1 gene of *T. gondii*, according to the technique developed by Costa et al. (2000) on the LightCycler Instrument.

The serum of the cow that aborted the fetus used for this study had a NAT titer of 1:10240 and no antibodies ($<$1:40) to *T. gondii*. Antibodies to *N. caninum* were detected in titers of 1:40 in 31 (29.2%), 1:80 in 22 (20.8%) and $\geq$1:160 in 23 (21.7%) of 106 cows from this herd.

4. Discussion

Mice are generally used to isolate viable *T. gondii* from aborted fetal tissues because they are more sensitive than cell culture methods (Dubey and Beattie, 1988). Unfortunately, outbred mice are not suitable hosts for the propagation of *N. caninum*. Immunosuppression with cortisone increases susceptibility of mice to *N. caninum* (Lindsay and Dubey, 1989b). Mice lacking the gene for interferon gamma (interferon gamma gene knock out) and nude mice have been used previously to isolate *N. caninum* from animal tissues (Dubey et al., 1998; Yamane et al., 1998); however, their ability to recover *N. caninum* from fetal calf tissues using these mouse strains is unknown. Additionally, knockout or nude mice are expensive to use in routine diagnosis. Desmonts and Remington (1980) described an ingenious method to increase growth of *T. gondii* tachyzoites in peritoneal cavities of mice co-infected with mouse sarcoma cells. Romand et al. (1998) adapted this technique to grow a large numbers of *N. caninum* tachyzoites for use in the *Neospora* agglutination test. In the laboratory at the University of Porto, *T. gondii* is grown routinely in mouse sarcoma cells in mice. This method can be suited for recovering *N. caninum* isolates from tissue samples: this isolation was achieved on our first attempt from an infected bovine fetus, which is also the first isolate of *N. caninum* in Portugal with genomic confirmation by PCR.

Antibodies to *N. caninum* were found in 71.6% of cows at a NAT titer of 1:40. The NAT titer that should be considered diagnostic for *N. caninum* infection has not been decided. Titers of 1:25, 1:40 and 1:80 have been suggested as cut-off values (de Marez et al., 1999; Venturini et al., 1999; Jenkins et al., 2000), therefore, we reported results from this herd at different titers.

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